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14. ABSTRACT

Results in the third grant year further support the "Unified Mechanism Hypothesis" in that: (1) Giving pregnancy associated hormones or hCG to virgin female rats either before or after MNU treatment elicits persistent serum AFP levels, thereby explaining why breast cancer appearance is inhibited when employing either treatment sequence; (2) Giving hCG to estrogenized SCID mice bearing human breast cancer xenografts inhibits the cancer growth, apparently by action of murine AFP that the treatment has elicited from the mouse liver as an inform with low avidity for our available anti mAFP antibody. We are able to detect mAFP in mouse serum by western blot when it is present in the very high concentration that is elicited by injection of the animals with high E3 doses. (3) In an "all-human" in vitro system, hCG elicits hAFP from cultured HepG2 human liver cancer cells, addition of the hAFP-containing supernate to cultures of MCF7 human breast cancer cells blocks their growth, and that adding anti hAFP antibody to that system prevents the inhibition. hAFP is thus confirmed as the proximal inhibitor.

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Table of Contents

Page

Introduction …….. 4

Body…….. 4

Key Research Accomplishments ……. 10

Reportable Outcomes…… 11

Conclusion …… 12

References …… 13

Appendices …….. None

INTRODUCTION

The substantial reduction of life-time risk for breast cancer that early term pregnancy confers on women has drawn the attention of researchers seeking new modalities for reducing the disease incidence among US women from the current level of 200,000 new cases/year. They have administered different pregnancy-associated hormones (steroid estrogens and/or progestins, or hCG) to carcinogen-treated virgin rats and have in most cases produced considerably reduced breast cancer occurrence.

We hypothesized that in each of these successful treatments the administered hormone has elicited secretion of AFP from the adult rat liver. This then appears in the serum, as it does in pregnancy, in which state AFP of fetal origin enters the maternal serum. We have shown in many studies that AFP is a potent anti-breast cancer agent. Our aims in this project have been (Aim 1) to show that the reduced incidence of breast cancer in carcinogen treated rats that earlier investigators produced by administration of hormones was accompanied in each case by the appearance of significant levels of AFP in the rat sera; (Aim 2) to show that administering a hormone of pregnancy to SCID mice bearing human breast cancer xenografts would elicit murine AFP into the serum which would inhibit the of growth xenograft; and (Aim 3) to show that in hormone-treated mice, these responses occurring at the protein level can be demonstrated as well at the genome level, by quantifying the induction of mRNA for AFP in mouse liver.

BODY

Additional Findings Relevant to Specific Aim 1:

Data we provided in earlier progress reports showed that among groups of MNU-treated female rats that were subsequently made pregnant or were administered hormones of pregnancy ($E_2 + P$, $E_3 + P$, E_3 or hCG), (1, 2, 3, 4, 5) fewer breast cancer appeared than among rats receiving MNU only. In accord with our hypothesis, western blot analysis of sera from these five groups at the mid point or at the end of the hormone treatment (or pregnancy), disclosed that significant levels of AFP had been secreted. We have now performed similar blots on sera taken one week after the final hormone treatment (or parturition) and in each case significant AFP levels still were present (Figure 1). This indicates that AFP persists for at least 7 days following cessation of the hormonal manipulations, and therefore accounts for the reported inhibition of breast cancer inhibition of breast cancer appearance when the carcinogen treatment follows the hormone treatment.

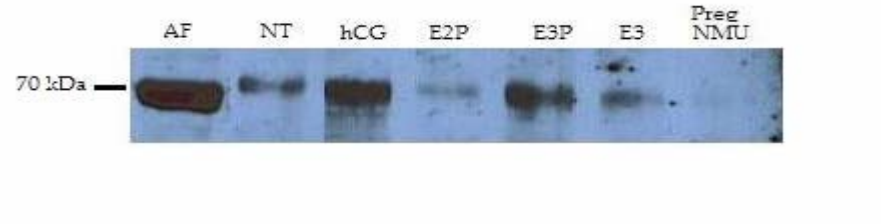
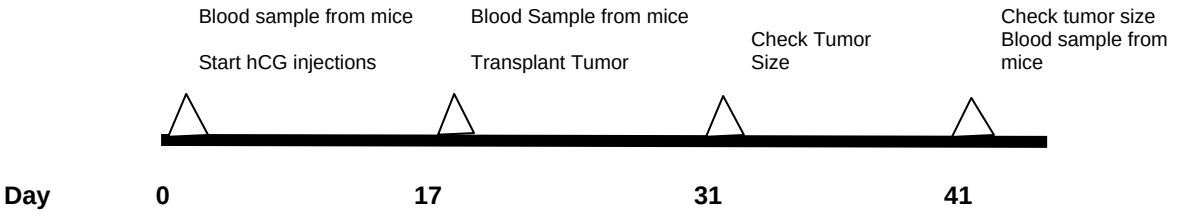


Figure 1. **Western Blots: AF in sera of NMU and hormone treated rats, at 7 days after hormone treatment ends.**

Specific Aim 2:
The aim is to show that growth of human breast cancer xenografts growing in estrogenized immunodefficient mice is inhibited or blocked by action of murine (m) AFP that injecting the animals with hCG would elicit from the adult liver. Further, mAFP would be found in sera of such mice. Additionally if this study were reproduced in mice that have been passively immunized against mAFP, inhibition of growth would not occur. This would demonstrate that the inhibition of human breast cancer xenografts growth was caused by its exposure to serum-born mAFP and not to some other substance elicited by the injection of hCG.
As shown in the study detailed below, the administration of hCG to the mice succeeded in inhibiting growth of human breast cancer xenografts. However, mAFP could not be detected in the human mouse sera with any anti mAFP Ab available to us and precluded any attempts for passive immunization against mAFP.

Aim 2, a. To evaluate the effect of hCG and its hypothesized mediator AFP on growing human breast cancer xenografts, the protocol described in Fig. 2 was employed.

Figure 2.



Severe Combined Immunodeficient (SCID) mice were divided into two groups, one designated to receive hCG and the other designated to receive vehicle for the duration of the experiment. hCG was given intraperitoneally once a day at a dose of 100 international units per mouse per day. This protocol was adopted from a report by Russo et al.(5), in which hCG had been shown to protect rats against development of DMBA- induced mammary tumors and verified by us that hCG protected rats against NMU-induced mammary tumors and induced AFP into blood. As shown in Figure 1, SCID mice were treated with hCG for 17 days prior to tumor implantation, a duration that was sufficient for induction of AFP into rat blood. For tumor implantation, human MCF-7 tumor xenografts growing in the mammary fat pad of donor SCID mice were harvested and cut into small pieces. Tumor pieces were carefully selected for homogeneous viable tumor, and transplanted under the kidney capsule of the vehicle-treated and hCG-treated SCID mice according to procedures previously published by our group (6). The subrenal capsule site was selected over the mammary fat pad or subcutaneous site because of the rapid and uniform rate of tumor growth in the subrenal capsule site (6). Tumor recipient mice also received subcutaneously an estradiol implant that provided a steady state level of 10^{-9} M estradiol in the blood (6). Treatment with vehicle or hCG were continued daily after tumor implantation. Tumor size was measured during survival laparotomy at the time of tumor implantation, 14 days after tumor implantation and at necropsy 24 days after tumor implantation. Blood samples were obtained prior to hCG treatment, at the time of tumor implantation, and at necropsy. As shown in Fig 2, treatment with hCG significantly inhibited tumor growth. In the vehicle treated group tumor volume increased almost 3-fold (180%), (Fig 2 A), whereas in the hCG-treated

group tumor volume increased by only 33%, (Fig 2 B). Thus in this experiment the overall inhibition of tumor growth by hCG was 60% (Fig 2 C).

Figure 2 A.

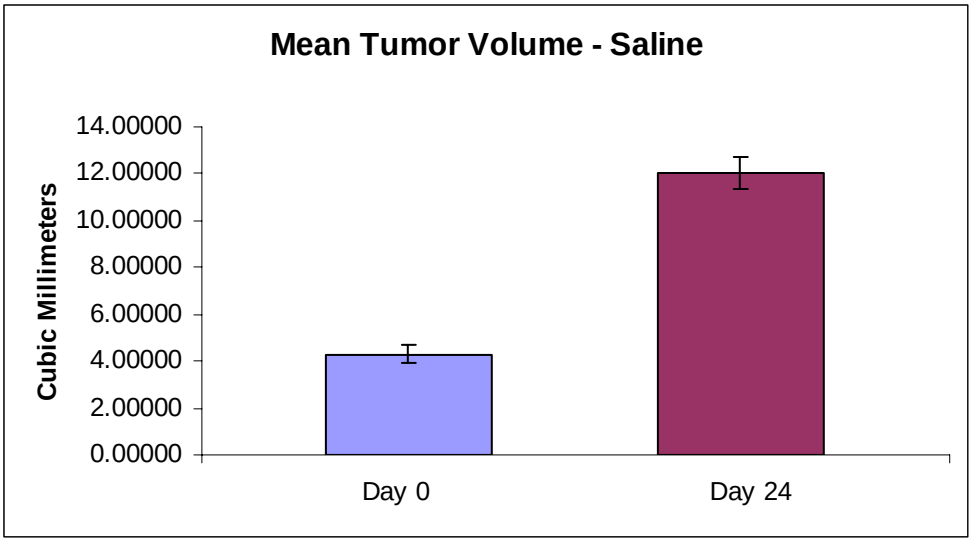


Figure 2 B.

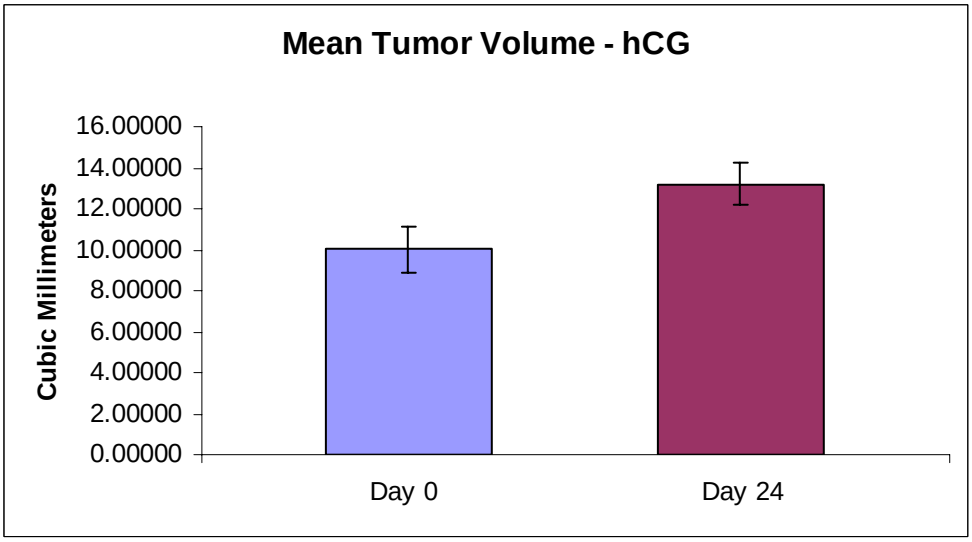
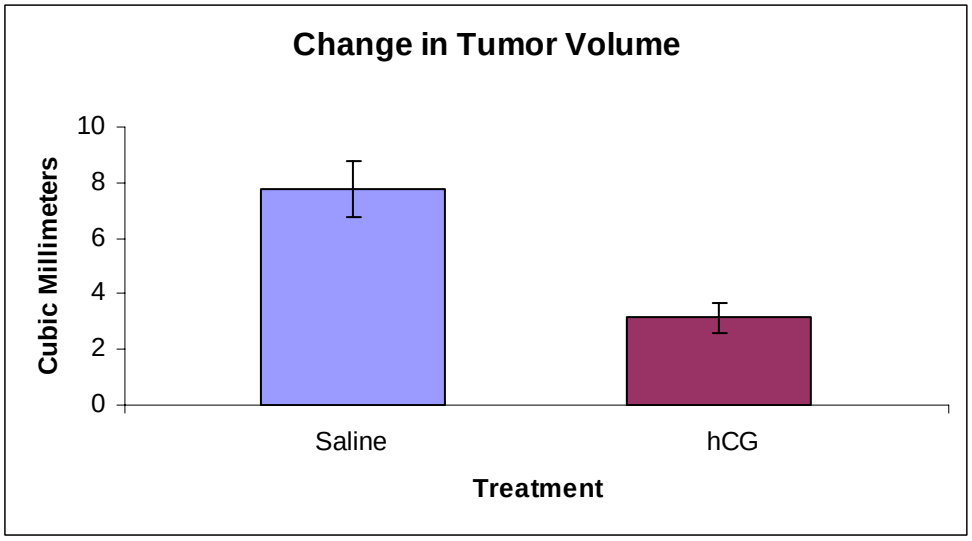


Figure 2 C.



Specific Aim 2, b.

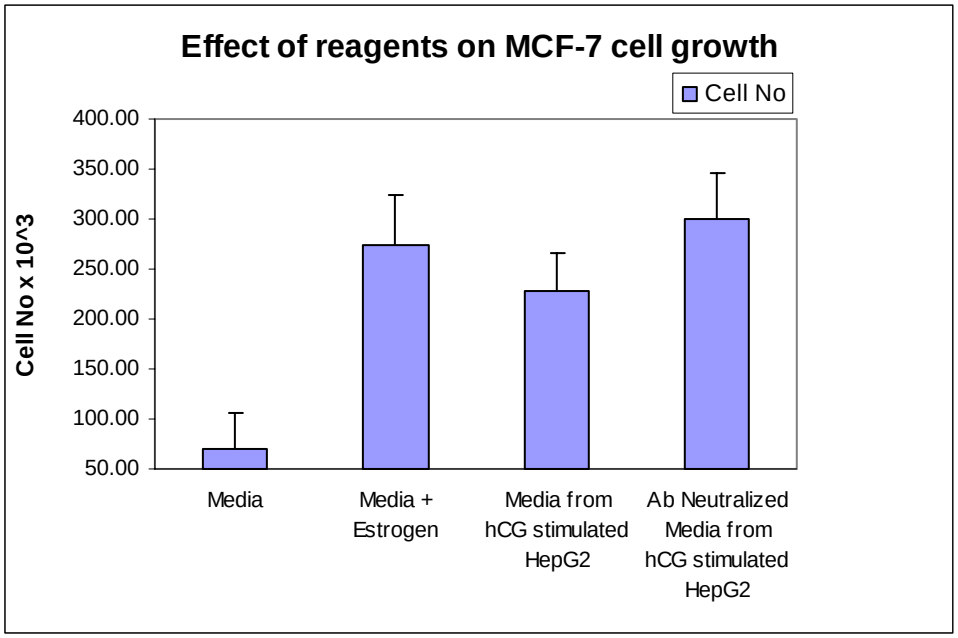
Western blot analysis of the mouse sera provided no evidence that hCG administration had elicited murine AFP from these animals, even though the hCG treatment had demonstrably inhibited growth of the breast cancer xenografts that they bore – an anticipated effect of AFP. We suspected that this apparent disconnect was due to the nature of the AFP antigen contained in the serum. Rodent AFP consists of a mixture of as many as 8 isoforms that differ primarily in the nature and burden of their glycosylation (7). We therefore tested the ability of our reagents to detect the form of murine AFP that appears in the sera of adult mice that have been hyper stimulated estriol (8).

We conclude that the apparent absence of mAFP in the hCG treated SCID mice reflects the inability of our antibody to react with that substance when it is present at extremely high concentration.

Clearly, using our available anti AFP antibodies we could not achieve passive immunization of mice against mAFP, the experimental step originally planned.

We pursued the objective of Specific Aim 2 by an alternate route, which would involve the use of a potent antibody to human AFP. This analogous model is an "all human" in-vitro system. In some, MCF-7 human estrogen dependant breast cancer cells are stimulated to grow in cell culture medium containing estradiol. Human HepG2 liver cancer cells are stimulated to secrete human AFP in culture medium containing hCG. Supernatant medium from these latter cultures, (containing hAFP), was introduced into the MCF-7 breast cancer cultures and blocked the E_2 stimulated growth. When this sequence was performed and anti hAFP antibody was added to neutralize the AFP, growth was accelerated. Conclusion: The principles we have proposed to establish in Specific Aim 2, have been demonstrated.

Figure 3.



Specific Aim 3.

In Specific Aim 3 we proposed to evaluate at the genome level the phenomena that we have been studying at the protein level. In a preliminary experiment we assessed the ability of high dose estriol treatment of male mice to provoke hepatic production of AFP, and in particular, to elevate hepatic content on mRNA for AFP (9).

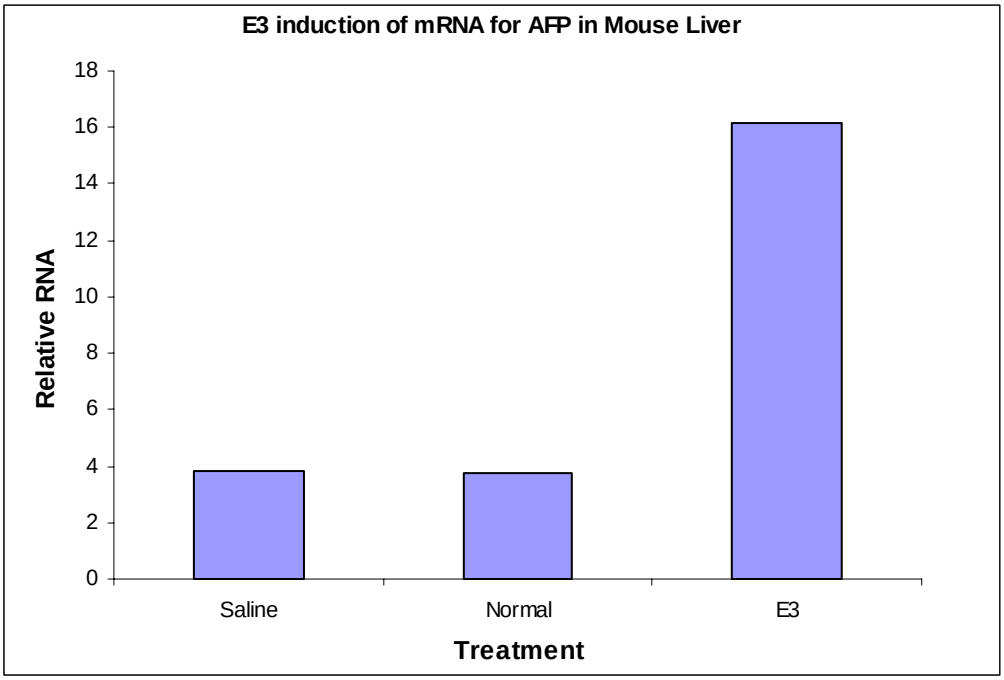
2 male BDF1 mice at 10 weeks of age were each injected i.p. with an aqueous suspension of 10 mg estriol in sterile saline containing 0.5 mg gum Arabic and 1.3 uL acetic acid (8). 1 mouse was injected i.p. with 1 ml sterile saline and 1 mouse was untreated.

After 7 days blood samples were drawn for AFP assay and at 14 days the mice were sacrificed and their livers were obtained. TRI Reagent (Molecular Research Center) was used to isolate total RNA from mice livers following manufacture's directions. RNA was reverse transcribed into cDNA using a reverse transcriptase (iScript cDNA Synthesis Kit from Biorad); the resulting cDNA was used as templates for subsequent PCR amplification using primers specific for mouse alpha-fetoprotein and B-actin as housekeeping gene. Primers were designed using Vector NTI. SYBR Green was used to detect amplification of the product and threshold cycles for each sample.

Relative abundance of AFP message was normalized to B-actin in each sample and calculated as 2-^(Ct AFP – Ct B-actin) x 1000, where Ct represents the threshold cycle for each transcript.

Results: Preliminary data based on samples from one mouse per each group show a four fold increase in AFP mRNA from the estriol treated mice in comparison to the saline treated and non treated mice.

Figure 4.


E3 induction of mRNA for AFP in Mouse Liver
Relative RNA
18
16
14
12
10
8
6
4
2
0
Saline
Normal
E3
Treatment

KEY RESEARCH ACCOMPLISHMENTS

- hCG treatment of SD rats elicits serum AFP levels that remain high 14 days after cessation of hCG treatment.

- E_2 + P4 treatment of SD rats elicits serum AFP levels that remain high 14 days after cessation of hCG treatment.

- $E_3 + P_4$ treatment of SD rats elicits serum AFP levels that remain high 14 days after cessation of hCG treatment.

- E_3 treatment of SD rats elicits serum AFP levels that remain high 14 days after cessation of hCG treatment.

- hCG treatment of estrogenized SCID mice bearing MCF-7 human breast cancer xenografts blocks the growth of the xenografts.

- Available anti mouse AFP Ab could detect murine fetal AFP (from amniotic fluid) at 100 ng/ml, whereas detecting adult murine isoform AFP from serum requires 100 times that concentration.

- Giving hCG to mice elicit hepatic mAFP that inhibits growth of human breast cancer xenografts.

- Available anti mAFP antibodies are inadequate for passive immunization of mice.

- In an "all-human" culture system: hCG stimulates human HepG2 to secrete hAFP into the culture media.

- Culture media containing hAFP added to cultured MCF-7 human cancer cells blocks their growth.

- Adding anti hAFP relieves the blockade: therefore hAFP is the sole cancer-inhibiting substance that was secreted.

- At the genome level, in high dose E_3-stimulated mice the mRNA for hAFP in liver is increased by 400%.

REPORTABLE OUTCOMES

1. Presentation in the CORE Curriculum, Department of Pathology, Albany Medical College.

2. Mentoring medical student who has been admitted to candidacy for the degree of Doctor of Medicine with Distinction in Research (MDDR).

3. Serve on three thesis committees for students working on projects related to this grant.

4. Presentation at the Fifth International Conference on Hormonal Carcinogenesis, Montpellier, France. Hormones of Pregnancy, AFP, and Reduction of Breast Cancer Risk. Herbert I. Jacobson, Nicole Lemanski, Amithi Narendran, Anu Agarwal, James A. Bennett and Thomas T. Andersen.

5. A peptide derived from alpha-fetoprotein inhibits the proliferation induced by estradiol in mammary tumor cells in culture. Walter D. Sierralta, Maria J. Epunan , Jose M. Reyes, Luis E. Valladares, Thomas T. Andersen, James A. Bennett, Herbert I. Jacobson, Ana M. Pino. Oncology Reports. In review.

*6. An alpha-fetoprotein derived peptide reduces the uterine hyperplasia and increases the anti-tumor effect of tamoxifen. Thomas T. Andersen, Justin Georgekutty, Lori A. DeFreest, Giyana Amaratunga, Amithi Narendran, Nicole Lemanski, Herbert I. Jacobson, James A. Bennett. British Journal Of Cancer. In Press 2007.

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Herbert I. Jacobson, Nicole Lemanski, Amithi Narendran, Anu Agarwal, James A. Bennett, and Thomas T. Andersen. in Hormonal Carcinogenesis V. J.J. Li and S.A.. Li, eds. Springer Verlag. In press, September 2007.

* Copies in Appendix

CONCLUSION

1) In carcinogen-exposed adult rats that are administered hormones to stop cancer appearance, AFP is elicited into the serum at levels that persist for at least 7 days after cessation of the preventive treatment. Conclusion: AFP is present and can account for the prevention of malignancies when hormones are given either before or subsequent to the carcinogen administration.

2) Treating estrogenized SCID mice that bear MCF7 human breast cancer xenografts with hCG blocks the growth of the tumor. Western blot examination of the murine serum did not detect mAFP, the putative proximal cancer inhibitor, because, as we found, the levels were below detectability by available antibodies. Available anti mouse AFP could detect murine fetal AFP (from amniotic fluid) at 100 ng/ml, whereas detecting the adult murine isoform of AFP elicited from adult liver into serum requires 100 times that concentration Conclusion: Giving hCG to mice elicits hepatic mAFP that inhibits growth of human breast cancer xenografts. (2) Clearly available anti mAFP antibodies are inadequate for passive immunization of mice.

3) As reported previously hCG stimulates hAFP secretion into the medium in cultures of human liver cancer cells (HepG2 line). Adding this supernatant medium to cultures of human MCF7 cancer cells blocks their growth. Adding anti hAFP Ab to that system neutralizes the hAFP and the cells grow; the inhibition has been prevented. This *in vitro* protocol indicates 1. the principle of the Unified Mechanism is valid for human tissues, and 2. that in the system the proximal inhibitor of breast cancer growth is hAFP and is not some other substance that hCG exposure might have elicited from the HepG2 cells.

4) As described in our recently granted request for a no-cost extension, we are proceeding to achieve at the genome level confirmation of the findings already obtained at the protein level.

SO WHAT

Our results show that the constellation of reports demonstrating inhibition of breast cancer that has been achieved in rats through administration of various pregnancy-associated hormones, have in fact all achieved that inhibition by eliciting the true proximal inhibitor, AFP, from the adult rat liver. We have shown AFP to have potent anti-breast cancer activity in many direct studies, and from our numerous epidemiologic and ecologic investigations we conclude that AFP is a breast cancer preventing agent for breast cancer in women. We believe that exploiting this biological property of the AFP molecule is the prime route to develop a practical modality for reducing breast cancer risk. We have parsed the 69,000 kilodalton AFP molecule to identify the region that is responsible for its activity. That active site is an 8 amino-acid sequence in the third domain of AFP. We have synthesized this peptide, termed AFPep, and have demonstrated its growth inhibitory activity against human breast cancers. We have modified this peptide to increase its activity as well as its stability in storage. It is active by the oral route. It inhibits breast cancers that have become resistant to tamoxifen. Its mechanism is different from that of tamoxifen and other drugs currently in use for the treatment of breast cancers. It blocks the uterine hyperplasia induced by tamoxifen and adds to the anti-breast cancer activity of tamoxifen. It prevents chemically induced breast cancers in rats. It has been synthesized under GMP conditions by an independent commercial company and that product was fully active. No evidence of toxicity has been found in mice, rats and dogs at doses up to one hundred times above the biologically active dose. The data support the conclusion that this AFP-derived peptide is a safe and effective new agent for the treatment and prevention of breast cancer and merits translation to the clinic.

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Appendix

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Full Paper

An α-fetoprotein-derived peptide reduces the uterine hyperplasia and increases the antitumour effect of tamoxifen

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Tamoxifen (Tam) is effective for the treatment and prevention of breast cancer. However, it has toxic drawbacks and has limited-duration utility because, over time, human tumours become refractory to Tam. Recently, a new nontoxic peptide, α-fetoprotein-derived peptide (AFPep) has been proposed for the treatment and prevention of breast cancer. The purpose of this paper is to determine whether combining AFPep with Tam would increase efficacy and reduce toxicity in experimental models of breast cancer. Low doses of AFPep and Tam were more effective in combination than either agent alone against breast cancer growth in cell culture, in tumour-xenografted mice, and in carcoinogen-exposed rats. α-Fetoprotein-derived peptide interfered with Tam-induced uterine hyperplasia in immature mice, and showed no toxic effects. Unlike Tam, AFPep did not inhibit binding of oestradiol (E_2) to oestrogen receptor (ER). Thus, these two agents utilise different mechanisms to interfere with ER functionality, yet work cooperatively to reduce breast cancer growth and alleviate Tam's troubling toxicity of uterine hyperplasia and appear to be a rational combination for the treatment of ER-positive breast cancer.
British Journal of Cancer (2007) **0,** 000–000. doi:10.1038/sj.bjc.6603882 www.bjcancer.com
© 2007 Cancer Research UK

Keywords: tamoxifen; AFPep; xenograft; uterine hyperplasia; cancer prevention; breast cancer; mechanism; toxicity

UNCORRECTED PROOF

A peptide (α-fetoprotein-derived peptide (AFPep), sequence *cyclo*(EKTOVNOGN), where O is hydroxyproline) derived from the active site of α-fetoprotein (AFP) has been under investigation as a potential agent for prevention or therapy of oestrogen receptor-positive (ER+) breast cancer (Mesfin *et al*, 2000, 2001; Bennett *et al*, 2002, 2006; DeFreest *et al*, 2004). After identifying the active site of AFP as an eight-amino acid peptide (Mesfin *et al*, 2000), Mesfin went on to develop a more stable, cyclised peptide with substantial potential as a pharmaceutical agent (Mesfin *et al*, 2001). α-Fetoprotein-derived peptide has been shown to inhibit the growth of human breast cancer xenografts in mice (Mesfin *et al*, 2001), and prevent the development of carcinogen-induced mammary cancers in rats (Parikh *et al*, 2005). It was also shown to inhibit the growth of breast cancer, which had become resistant to the cytostatic effects of tamoxifen (Tam; Bennett *et al*, 2002). α-Fetoprotein-derived peptide is active after oral administration (Bennett *et al*, 2002), and has not exhibited toxicity in any study to date.

Tamoxifen has been the most widely used and effective drug for the treatment of ER+ breast cancer for many years (Jordan, 1999a). It has been shown to inhibit breast cancer growth (Lippman and Bolan, 1975), inhibit breast cancer recurrences (Early Breast Cancer Trialists' Collaborative Group, 1992), and decrease the risk of primary breast cancers in high-risk patients (Fisher *et al*, 1998). However, as mentioned above, some ER+ breast cancers acquire resistance to Tam, and some are actually resistant to Tam before treatment (Jensen and DeSombre, 1996). Moreover, although Tam is relatively well tolerated, it is not without unwanted sequelae in some patients. These toxicities and side effects are generally dose-dependent and include uterine hyperplasia, which can progress to uterine cancer in a small percentage of patients; thrombo-embolic episodes that can progress to deep vein thrombosis, pulmonary embolism, and stroke in a small percentage of patients; and nonlife-threatening side effects such as hot flashes, fluid retention, and vaginal discharge (Mosby, 2005). Reducing Tam-induced toxicity and providing alternatives to Tam for Tam-resistant tumours would advance the treatment of breast cancer. We have been investigating AFPep for these purposes (Mesfin *et al*, 2000, 2001; Bennett *et al*, 2002, 2006; DeFreest *et al*, 2004; Parikh *et al*, 2005). However, as part of this investigation, it seemed reasonable to evaluate AFPep in combination with Tam in that it might add to the therapeutic activity of Tam, and perhaps might even reduce the toxicity or side effects of Tam. This report describes results of AFPep in combination with Tam in models of breast cancer therapy, prevention, and host toxicity.

MATERIALS AND METHODS

Materials

Carcinogen (*N*-methyl-*N*-nitrosourea, MNU) was obtained from the National Cancer Institute carcinogen repository (MRI Inc., Kansas City, MO, USA) and was dissolved in sterile physiological saline (1%, w/v), buffered to pH 5.0 with 3% acetic acid. Female

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Computational Design and Experimental Discovery of an Antiestrogenic Peptide Derived from α-Fetoprotein

Karl N. Kirschner,*,† Katrina W. Lexa,† Amanda M. Salisburg,† Katherine A. Alser,†
Leroy Joseph,‡ Thomas T. Andersen,*,‡ James A. Bennett,§
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Abstract: Breast cancer is the most common cancer among women, and tamoxifen is the preferred drug for estrogen receptor-positive breast cancer treatment. Many of these cancers are intrinsically resistant to tamoxifen or acquire resistance during treatment. Consequently, there is an ongoing need for breast cancer drugs that have different molecular targets. Previous work has shown that 8-mer and cyclic 9-mer peptides inhibit breast cancer in mouse and rat models, interacting with an unsolved receptor, while peptides smaller than eight amino acids did not. We show that the use of replica exchange molecular dynamics predicts the structure and dynamics of active peptides, leading to the discovery of smaller peptides with full biological activity. Simulations identified smaller peptide analogues with the same conserved reverse turn demonstrated in the larger peptides. These analogues were synthesized and shown to inhibit estrogen-dependent cell growth in a mouse uterine growth assay, a test showing reliable correlation with human breast cancer inhibition.

Introduction

Breast cancer is the most common cancer diagnosed in women and is the second leading cause of cancer death among women, closely following lung cancer. In 2006, the American Cancer Society estimated that 212 920 women in the United States will be diagnosed with invasive breast cancer and predicted 40 970 deaths.[1] Tamoxifen is the most widely used drug for the treatment of estrogen receptor-positive breast cancer, acting through competition with estrogen for binding to the estrogen receptor (ER).

However, approximately one-third to one-sixth of ER-positive breast cancers are intrinsically resistant to tamoxifen, and many more acquire resistance to this drug during treatment.[2] Additionally, tamoxifen stimulates uterine growth, which can lead to uterine cancer in a small percentage of women taking this drug.[2] Consequently, there is an ongoing need for breast cancer drugs with greater efficacy and fewer side effects.

α-Fetoprotein (AFP) is an embryo specific serum α-globulin glycoprotein that is synthesized primarily by the fetal liver and circulates through the serum of pregnant women.[3] AFP has been reported to bind and transport ligands, including fatty acids, steroids, heavy metal ions, phytoestrogens, drugs, and some toxins.[4] AFP is a growth-regulating hormone with the capacity to stimulate or inhibit growth.[5] From fertilization through birth, AFP holds an important physiological role as a developmental promoter for the fetus. More recent reports have shown that AFP has antiestrogenic activity and can inhibit the growth of estrogen dependent cancer.[2,6−8] These data, combined with epidemiological data showing that elevated levels of AFP are associated with a reduced lifetime risk of breast cancer,[7] have led to the suggestion that AFP or analogues thereof may be a useful agent for chemoprevention, as well as for the treatment, of breast cancer.[7,9,10] Festin et al.,[11] Eisele et al.,[12,13] and Mesfin

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et al.[14] parsed the 70 000 MW AFP into a series of smaller peptides that retained the same antiestrogenic and antibreast cancer activities of the original protein. Their work resulted in the smallest known active analogue, an 8-mer with the sequence EMTPVNPG (AFPep),[14] which consists of amino acids 472−479 from the human AFP sequence. Amino acid substitution studies revealed the importance of specific residues essential for activity.[15] All efforts since then to create an active peptide consisting of fewer than eight residues has resulted in the loss of antiestrogenic activity.[14,15]

A receptor for AFP on cancer cell membranes has been reported,[16] and binding of AFP (or AFPep) to this receptor signals the cell to inhibit its own growth. The steric/electronic features of the receptor site that permits AFP binding are largely unknown, making rational development of lead compounds difficult. We present here a novel strategy for developing new lead compounds, a strategy that uses molecular dynamics to explore the allowed conformational space of potentially active analogues in solution. Rational drug design using molecular dynamics in this manner involves understanding the conformational space occupied by the active compounds followed by the creation of different compounds that sample the same space. We have used Replica Exchange[17,18] Molecular Dynamics (REMD) techniques[19] to explore the conformational dynamics of several AFPep analogues. Our REMD results reveal that the peptide's critical region for activity is a four amino acid sequence that adopts a Type I β-turn conformation. We have run REMD simulations on several different four and five amino acid peptides, synthesized those that appeared promising, along with controls, and tested them for activity using an immature mouse uterine growth assay.[2] Results from the REMD simulations and the experimental activity studies are presented in this paper and show that peptides as short as four amino acid residues retain biological activity.

The REMD technique has been successfully used to obtain energy landscapes for the TRP cage and for the C-terminal β-hairpin of protein G,[20−22] to find the global minimum for chignolin[23] and to explore unfolding of α-helical peptides as a function of pressure and temperature.[24] REMD has also been used to explore intrafacial folding and membrane insertion of designed peptides[25] as well as simulations of DNA.[26,27] Recent work has focused on use of REMD to determine peptide structure and peptide and protein folding pathways.[28−85] We

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demonstrate for the first time that REMD, besides being an excellent technique for probing peptide and protein structure, can be used for a lead compound design.

Methods

We used the AMBER 8 molecular dynamics program package[19,86] with a new force field that has been created specifically to improve the representation of peptide structure.[87] Simulations were run in implicit solvent, using the Generalized Born (GB) model[88] implemented in AMBER[89] with the default radii under the multi*sander* framework.[89] It has been demonstrated that the use of continuum solvent models in REMD can lead to overstabilization of ion pairs that affect the secondary structure,[90,91] but since we do not have salt bridges in our peptide structure we avoid this potential problem. Adequate sampling of conformational space was insured through the use of the Replica Exchange algorithm.[17,18] REMD was developed as a means to overcome local potential energy barriers. Compiled under AMBER 8, one-dimensional REMD explores a generalized canonical ensemble of N noninteracting replicas that undergo simulation separately but concomitantly at exponentially related temperatures, with exchanges between replicas occurring at a specified time interval. The consequence of this exchange is that entrapment in local potential energy wells is avoided. Those replicas that do become trapped within a local well at one temperature can escape when transitioned to a higher temperature as part of the exchange process. Thus, accuracy of conformation is maintained through analysis at low temperatures, while simulations at higher temperatures efficiently achieve exploration of the potential energy surface.

The sequences used for the simulations were chosen from the set of previously synthesized active analogues.[15] REMD simulations were run on the cyclic analogues cyclic-[EKTPVNPGN], cyclic-[EKTPVNPGQ], cyclic-[EMTPVNPGQ], and the linear analogues EMTPVNPG and EMTPTNPG. In addition, REMD simulations were run on the smaller analogues EMTPVNP, MTPVNPG, TPVNP, TPVN, and PVNP. All sequences were capped using an acetyl beginning residue and an *N*-methylamine ending residue. Eight different replicas were used, each defined initially with the same input structure. Temperatures were selected to agree with exponential growth such that interchange occurred within the temperature group $temp_i$, $temp_o = 265$, 304, 350, 402, 462, 531, 610, and 700 K. The same temperatures were used for each peptide simulated and controlled using a weak-coupling algorithm as specified by ntt = 1. The number of exchange attempts between neighboring replicas was set to 1000, and the number of MD steps between exchange attempts was defined as 10 000. Thus, the total length for each simulation with a time step of 0.002 ps was 20 ns.[19] Additional methodological information can be found in a forthcoming paper.[92]

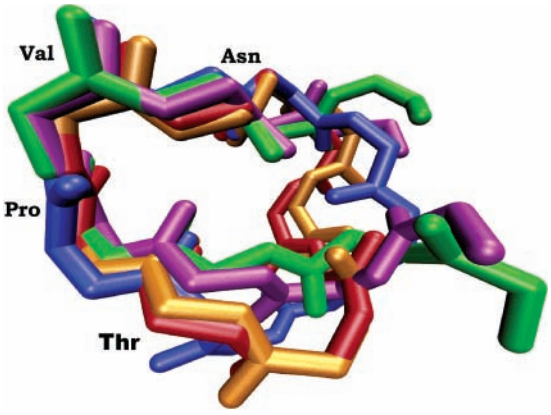

Figure 1. Overlay of the cyclic-[EKTPVNPGN] (red), cyclic-[EKTPVNPGQ] (blue), cyclic-[EMTPVNPGQ] (orange), and the EMTPVNPG (green) and EMTPTNPG (purple) peptides from REMD simulations. Each structure represents the β-turn motif sampled during the dynamics.

The linear peptide analogues shown in Table 2 were prepared using Fmoc solid-phase synthesis.[14,15,93] The antiestrogenic activity of each peptide was determined using the immature mouse uterine growth assay as described previously.[2,7] Intraperitoneal (i.p.) administration of 0.5 μg of 17β-estradiol (E_2) to an immature female mouse doubles its uterine weight in 24 h.[7] Swiss/Webster female mice, weighing 6−8 grams at 13−15 days old, were obtained from Taconic Farms. Mice were grouped so that each group had the same range of body weights. Each group received two sequential i.p. injections spaced 1 h apart. The peptide or a saline solution control was contained in the first injectant, and E_2 or a saline control was contained in the second injectant. 22 h after the second injection, uteri were dissected and weighed immediately. The uterine weights were normalized to mouse body weights (mg of uterine/g of body). Experiments used a minimum of five mice per group, and the mean normalized uterine weight and standard deviations were determined for each group. Percent growth inhibition in a test group was calculated from the normalized uterine weights as given by eq 1.

$$\text{Growth inhibition (\%)} = (\text{Full } E_2 \text{ stimulation} - E_2 \text{ stimulation in test group})/(\text{Full } E_2 \text{ stimulation} - \text{No } E_2 \text{ stimulation}) \times 100\% \quad (1)$$

The significance of differences between groups was evaluated with the nonparametric Wilcoxon ranks sum test (one-sided). Generally, drug-induced growth inhibitions of 20% or greater are significantly different ($p \leq 0.05$) from the group receiving E_2 alone. Each AFPep analogue was evaluated for antiuterotrophic activity in three or more experiments, and the mean growth inhibition ± the standard error for each analogue is reported in Table 2.

Results

Figure 1 shows the conservation of conformational space of the five active 8-mer and cyclic 9-mer AFP-derived peptides previously shown[14,15] to have antiestrogenic activity. This figure shows the overlay of the most representative peptide geometries obtained from the conformational family that displays the common reverse turn motif in each REMD simulation. Four amino acids, TPVN, are conserved in the conformational space of the active 8-mers and cyclic 9-mers. The TPVN sequence forms a reverse turn within the longer peptides, a structure that is conserved across all five REMD simulations. In a Type I reverse β-turn, four amino acids form a turn structure, which is

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Table 1. Average ϕ/ψ Angle Values for the Second and Third Amino Acids for Five Different Sequences[a]

		ϕ_2	ψ_2	ϕ_3	ψ_3
type I β-turn		−60	−30	−90	0
cyclic-[EM**TPVN**PGQ]		−68	−16	−96	−9
EM**TPVN**PG[b]		−68	−17	−94	−11
TPVNP		−65	−19	−95	−12
TPVN		−68	−19	−88	−11
PVNP	68%	−89	−8	−110	115
	27%	−108	141	−104	128

[a] The four residues in boldface define amino acids one through four for each peptide. The ϕ/ψ angle values for the second and third amino acids of a four amino acid sequence are diagnostic for a β-turn structure. [b] The sequence of the AFPep peptide.

defined by the phi (ϕ) and psi (ψ) angles of the second and third amino acids. An ideal Type I β-turn has ϕ/ψ values of −60° and −30° for the second amino acid and ϕ/ψ values of −90° and 0° for the third amino acid in the four amino acid turn sequence. As displayed in Table 1, the cyclic-[EMTPVNPGQ] and EMTPVNPG peptides have average ϕ_{Pro}/ψ_{Pro} and ϕ_{Val}/ψ_{Val} values that fall within 13° of a Type I turn. The other three simulations of the 8-mer and 9-mer peptides have similar values (Figure 1).

We ran REMD simulations for the TPVN, TPVNP, and PVNP analogues to explore the conformational space sampled by these four and five amino acid peptides. The TPVN and TPVNP peptides form the same reverse turn seen in the larger, active peptides. As displayed in Table 1, the TPVNP and TPVN analogues have ϕ_{Pro}/ψ_{Pro} values of approximately −67° and −19°, while the ϕ_{Val}/ψ_{Val} values are approximately −91° and −12°. In contrast, the average ϕ_{Val}/ψ_{Val}, and ϕ_{Asn}/ψ_{Asn} values for the two dominant conformations of the PVNP peptide reveal that none of the three structures is a turn (Table 1). The structure that is sampled for 68% of the REMD simulations has ϕ_{Val}/ψ_{Val} angles of −89° and −8° and ϕ_{Asn}/ψ_{Asn} angles of −110° and 115°. The structure sampled for 27% of the REMD simulation has ϕ_{Val}/ψ_{Val} angles of −108° and 141° and ϕ_{Asn}/ψ_{Asn} angles of −104° and 128°. Consideration of the average asparagine angles alone shows that the PVNP peptide does not form a turn structure.

The representative dynamics of three analogues containing the conserved TPVN sequence can be visualized in Figure 2. The top graphs display the distance between the C_α atoms of the threonine and asparagine residues, serving as a definitive diagnostic for a β-turn. Twenty-five percent of β-turns do not have an intraturn hydrogen bond, so an alternative definition of a β-turn is that the distance between the C_α carbon atoms of amino acid residues one and four in the tetrapeptide sequence is less than 7 Å.[94] The TPVN tetrapeptide has a $C_{\alpha(T)} - C_{\alpha(N)}$ distance less than 7 Å for 64% of the simulation, indicating a β-turn. The TPVNP pentapeptide adopts the turn structure for 74% of the simulation, and the cyclic-[EMTPVNPGQ] peptide is locked into the turn structure for 99% of the simulation. The bottom graphs in Figure 2 show the corresponding three-dimensional plots of ϕ versus ψ. These plots reveal the dynamics of the ϕ/ψ values for proline (red) and valine (green) amino acids throughout the simulation for these three peptides. These plots confirm that these three peptides adopt a β-turn conformation over the course of the simulation.

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Several 4-mer and 5-mer peptide analogues containing the TPVN sequence, or a similar sequence with hydroxyproline (O) substituted for proline, were synthesized, tested, and compared to the original 8-mer peptides for biological activity. As shown in Table 2, biological activity, as defined by inhibition of estrogen-stimulated growth of an immature mouse uterus, was retained in the TPVN and TOVN 4-mers and even more so in the TPVNP and TOVNO 5-mers.

The OVNO and PVNP analogues, which were thought to represent the pharmacophore region of AFPep,[15] did not show significant activity. Similarly, five amino acid peptides containing the amino or carboxyl end of AFPep did not have biological activity (bottom of Table 2). The 7-mer, EMTPVNP, was slightly less active than AFPep and the smaller TPVNP.

The figures reveal why the TPVNP and TPVN analogues are active. All active peptides have a conserved reverse β-turn motif. These β-turns are formed by the TPVN sequence, with a hydrogen bond formed between the carbonyl oxygen of the first amino acid and the amide hydrogen of the fourth amino acid. The conservation of proline in the second position favors the formation of a reverse turn. This proline is conserved in human, gorilla, chimpanzee, horse, rat, and mouse AFP sequences.[15]

Discussion

We have used REMD simulations to sample the conformational space of 8-mer and 9-mer AFP-derived peptides that have antiestrogenic and antibreast cancer activity. We discovered that an identical four amino acid sequence had minimal conformational flexibility, suggesting that this region is essential for the biological activities of these peptides. The TPVN, TOVN, TPVNP, and TOVNO sequences were subsequently synthesized and were found to be biologically active. This is a novel finding because Mesfin et al.[11] and DeFreest et al.[15] had concluded that the 8-mer, EMTPVNPG, was the minimum number of residues that an AFP-derived peptide can have and still retain significant antiestrogenic activity. This conclusion was based on the findings that the 7-mers, MTPVNPG and EMTPVNP, had relatively less biological activity than the 8-mers. These investigators concluded that the 8-mer peptide assumed a pseudo-cyclic conformation, that the middle of the peptide contained its pharmacophore region (the PVNP sequence), and that the exterior residues were essential for holding the peptide in its active conformation. The 7-mers lost activity, which was assumed to result from the loss of hydrogen bonding between the first and eighth amino acid residues, suggesting that full activity must require at least eight amino acid residues. Indeed the 5-mer, EMTPV, was synthesized and found to have no significant activity, which supported their conclusion that the 8-mer sequence was the minimal sequence for biological activity.[14] Furthermore, guided by this hydrogen bond hypothesis, cyclic 9-mers were also synthesized and shown to possess significant biological activity.[93] We tested this pseudocyclic conformation hypothesis by examining the percentage of hydrogen bond formation between the first and eighth amino acids for each simulation containing seven or eight amino acid residues. For all 8-mer and 7-mer simulations, hydrogen bond formation between the first and the last amino acids is observed for less than 1% of the simulation if the distance definition for a hydrogen bond is set at 2.3 Å. This result is contrary to the original hypothesis.

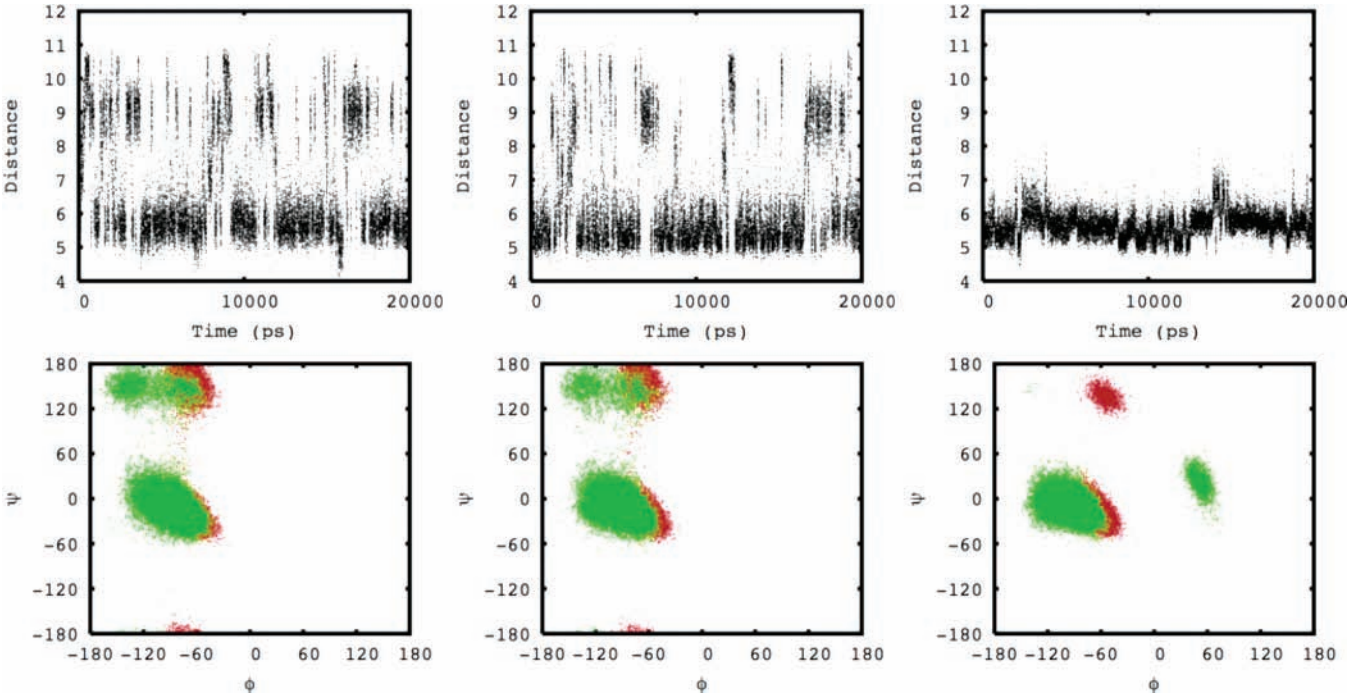

Figure 2. The top graphs depict the distances between the conserved threonine and asparagine C_α atoms as a function of simulation time for the TPVN (left), TPVNP (middle), and cyclic-[EMTPVNPGQ] (right) peptides. The bottom graphs depict their corresponding ϕ (*x*-axis) and ψ (*y*-axis) angles as a function of simulation time for the conserved proline (red) and valine (green) amino acids.

Table 2. Effect of AFP-derived Peptides on Estrogen-stimulated Growth of Immature Mouse Uterus[a]

sequence	% inhibition ± SE
EMTPVNPG	34 ± 3[b]
EMTOVNOG	32 ± 4[b]
EMTPVNP	19 ± 2
TOVNO	31 ± 4[b]
TPVNP	26 ± 1[b]
TPVN	24 ± 5[b]
TOVN	22 ± 1[b]
OVNO	6 ± 5
PVNP	5 ± 3
PGVGQ	0
EMTPV	0
EMTOV	0

[a] Estradiol and peptide injection into immature mice was carried out as described in Methods. Peptide was injected intraperitoneally at a dose of 1 μg per mouse. [b] $p < 0.05$ when compared to group stimulated with E_2 alone. Wilcoxon Rank-Sum Test.

The REMD studies reported herein lead to a substantially different conclusion regarding the requirement for peptide activity. These studies indicate that the key region is the TPVN sequence, since this structure retains the turn conformation common in every active peptide; the PVNP peptide does not sample a reverse turn structure (Table 1) and shows insignificant activity (Table 2). The above discovery has profound consequences. First, a 4-mer or a 5-mer peptide that retains biological activity is less expensive to synthesize than an 8-mer or a 9-mer peptide, and these smaller analogues are more druglike. Second, knowing the conformational space occupied by the 4-mer provides insight into the topology of the unknown receptor site for these peptides. Based on this study, the receptor site topology for the AFP analogue peptides is predicted to be a mirror image of a reverse β-turn. We note that the β-turn solution conformation of these peptides may not be retained when bound in the active site. However the high correlation between activity and β-turn conformation coupled with the entropic cost for altering the conformation upon binding gives us confidence that the receptor topology will accommodate a β-turn conformation. Previous substitutions in the 8-mer sequences reveal that when threonine, leucine, or isoleucine is substituted for valine, biological activity is retained, while substitution of valine with D-valine or alanine results in loss of activity. This implies that the topology of the receptor is stereospecific, and branched amino acids are essential for creation of hydrophobic forces that bind the receptor to the peptide. Finally, it leads to a different conceptual approach for stabilization of these peptides and the development of peptidomimetics. Peptidomimetics can be designed based on the active 4-mer and 5-mer peptides, with REMD used to ensure that the steric and electronic nature of the peptides is retained. Developing a peptidomimetic may not be necessary, for as long as the peptides are bioavailable they have the advantage of a lower probability of side effects compared to peptide mimics. The AFP 8-mer and cyclic 9-mer peptides have already been shown to be nontoxic and bioavailable in mice.[2] Cancer xenograft assays in mice involved the administration of 8-mer and cyclic 9-mer peptides twice a day for 30 days, during which time tumor growth was significantly inhibited, and there was no change in mouse body weight, cage activity, fur texture, or body temperature. In addition there were no changes in size or appearance of major organs relative to the control group. The uterine growth studies revealed that these peptides, unlike tamoxifen, did not stimulate murine uterine growth; indeed they inhibit the uterine stimulated growth induced by tamoxifen.[2,8] Thus, the peptides derived from AFP represent a new class of potential breast cancer drugs, which are active through a new, yet to be discovered, receptor.

Because there is excellent correlation between the uterine growth assay and the human breast cancer xenograft assay with regard to AFPep peptide inhibition of estrogen-stimulated growth,[2,14] the TOVNO, TPVNP, TOVN, and TPVN analogues

that are all active in the uterine growth assay are predicted to inhibit human breast cancer growth. We have begun the xenograft assays on these analogues to evaluate this prediction.

Conclusions

We have applied and demonstrated for the first time that REMD simulations can be used as a novel lead compound design tool. We have shown that REMD predicts a common conformation that is shared between the active linear 8-mer and cyclic 9-mer peptides. The predicted common conformation is a conserved reverse β-turn, and the smaller peptide analogues TOVNO, TPVNP, TOVN, and TPVN also contain the same conserved reverse turn. These analogues are shown to inhibit estrogen-dependent cell growth in a mouse uterine growth assay, through interaction with a yet to be discovered key receptor, and are predicted to inhibit human breast cancer. The 5-mer and 4-mer peptides are new discoveries that may lead to promising new antibreast cancer drugs.

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34

Hormones of Pregnancy, AFP, and Reduction of Breast Cancer Risk

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Summary

Background. Parity profoundly reduces the risk of acquiring breast cancer later in life. It has been reasoned that hormones (either estradiol E_2 or estriol E_3), progesterone (P), or human chorionic gonadotropin (hCG) in the serum of pregnant women might lead to that reduction in risk. These agents have been shown to reduce breast cancer incidence in non-pregnant rats. We investigated the hypothesis that exogenously added E_2, E_3, P, or hCG are not the proximal effectors of risk reduction, but that they elicit α-fetoprotein (AFP) from the non-pregnant liver, and that AFP is the proximal agent by which reduction of breast cancer risk is obtained. **Methods.** Methylnitrosourea(MNU)-exposed animals were treated with saline, E_3, E_2 + P, E_3 + P, hCG, or were allowed to experience pregnancy, and AFP levels were measured in the serum and subsequent tumor incidence was recorded. Human HepG2 liver cells in culture were treated with E_3, E_2 + P, P, or hCG and elicited AFP was measured in the media. The HepG2 culture media containing elicited AFP was assessed for its ability to inhibit proliferation of T47D cells when applied to these human breast cancer cells in culture, and to inhibit the estrogen-induced phosphorylation of the estrogen receptor in T47D cells. **Results.** For each condition in the prevention studies, hormone treatment reduced the incidence of breast cancer to an extent similar to that reported by the original studies. In each condition, AFP levels in serum were elevated over that in control animals. In culture, treatment of human liver cells with E_3, E_2 + P, or hCG, but not P alone, led to increased levels of AFP in the media. Media containing hCG-elicited AFP inhibited the estrogen-stimulated proliferation of T47D cells in culture, and inhibited phosphorylation of the estrogen receptor, whereas, estrogens and hCG did not inhibit the growth of these tumor cells in culture. **Conclusion.** Since the hormones of pregnancy elicit AFP from the liver, and AFP but not the hormones of pregnancy has direct anti tumor properties, it is concluded that AFP is the proximal agent through which reduction in breast cancer incidence is realized from the experience of pregnancy.

Introduction

Early parity profoundly affects women's risk of acquiring breast cancer, reducing it to about half that of nulliparous women (1). What is the mechanism? Can it be developed as therapy? Researchers seeking modalities for reduction of breast cancer risk have reasoned that some component of the serum during pregnancy must be an effector of the reduction in risk; posited effectors are the steroidal estrogens (estradiol E_2 or estriol E_3) (2, 3), progesterone (P) (2, 3), and human chorionic gonadotropin (hCG) (4). Additionally, alpha-fetoprotein (AFP) has been posited to be the risk-reducing agent (5).

The carcinogens MNU and dimethylbenzanthracene (DMBA) are potent generators of breast cancer in Sprague Dawley rats. Carcinogen-exposed female rats that are subsequently mated and bear litters ultimately generate half as many tumors as those that remain nulliparous (6). This striking parallel to the effect of pregnancy on human breast cancer risk (1) has suggested studies of cancer yield to be performed in carcinogen-exposed rats that have been administered pregnancy-associated hormones as surrogates for pregnancy. In such studies, Russo's laboratory (4) administered hCG, Lemon (7) and subsequently others

used E_3, and Grubbs (2) , Nandi, et al (3) and others have employed combinations of steroid hormone (P + either E_2 or E_3). Interestingly, all of these challenges produced similar results – a reduction of breast cancer incidence among carcinogen-exposed rats. We are aware of no hypotheses that would explain the same result from all of these various surrogates. There is need to account for this apparent redundancy of mechanisms available to the mammary gland for protection against malignant transformation.

We have studied the role of AFP in the parity-risk phenomenon, and have noted as well that this protein is an effective inhibitor of human breast cancer xenograft growth (8). We identified the active site of the 69,000 kD AFP molecule, synthesized a 9-mer cyclic peptide analog, AFPep, and have shown that it is effective for the prevention of breast cancer in MNU-treated rats (9), as well as for the inhibition of breast cancer xenograft growth. We hypothesize that E_3, E_2 + P, E_3 + P and hCG may be effective cancer preventive agents by virtue of their ability to elicit AFP secretion from the non-pregnant rat liver, rather than by having a direct effect on the mammary gland, and that AFP is the proximal agent by which the reduction in breast cancer risk is obtained. To test that hypothesis, we have repeated the hormone treatments that were reported to be effective at reducing rat breast cancer, in the same experimental configuration used by the previous researchers, and have measured serum AFP levels in these carcinogen-exposed, hormone-treated animals. Further, as a surrogate for human liver, we examined the ability of these hormones to elicit AFP from human liver cells in culture. We report here that these hormone treatments do, in fact, lead to the production of AFP and that hormone-elicited AFP directly inhibits growth of breast cancer cells in culture.

Materials and Methods

Carcinogen Exposure. Groups of 30 female Sprague Dawley rats received 50 mg/kg body weight of MNU in sterile physiological saline at 50 days of age as a single intra-jugular vein injection. Treatment with hormones was initiated within 10-21 days after MNU exposure and for each experiment continued for the durations used by the earlier workers. For tumor detection, animals were palpated twice weekly, continuing for 123 days.

Hormone Treatments. These followed the doses and schedules specified in the publications being replicated, and therefore differ from one another in modest ways. In every case, we used MNU, though some of the published investigations used DMBA. **E_2 + P** Following Grubbs *et al*. (2), ten days after carcinogen administration, rats received 20 μg E_2 plus 4 mg P dissolved in sesame oil, daily, by subcutaneous injection (0.2 ml), for 40 days. **E_3 + P** Following Rajkumar *et al*. (3), thirteen days after MNU, and under isofluorane anesthesia, rats received two individual subcutaneous Silastic capsules, one packed with 30 mg E_3 and another packed with 30 mg of P. These were left in place for 21 days to mimic pregnancy. **E_3** Following Rajkumar *et al*. (3) thirteen days following MNU administration, under isofluorane anesthesia, rats received a subcutaneous Silastic capsule packed with 30 mg E_3, implanted subcutaneously that remained in place for 21 days to mimic pregnancy. **hCG** Similar to Russo *et al*. (4), twenty-one days after MNU administration, rats received 100 IU hCG in de-ionized water, ip daily, for 60 days. **Pregnancy.** Following Grubbs *et al*. (11), ten days following MNU, female rats were introduced to males. Females stayed with males for 7 days, then were removed and separated into individual cages. Twenty-one to twenty-three days later, 19 females bore litters and were allowed to breast feed for 15 days. Females that did not become pregnant were excluded from the study. **Controls**. The No-Treatment group was exposed to MNU without subsequent treatment, and experienced the maximal number of tumors over the course of the ensuing four months. **Blood Samples.** Tail vein blood was drawn from animals in each of the 6 groups of rats at four time points: prior to first dose of treatment (for pregnancy, on the fourth and final day females were housed with males), at the midpoint of the treatment regimens, on the last day of the treatment regimen, and lastly, 14 days later. Blood was allowed to clot at room temperature, centrifuged, and sera stored at -20^{o}C. No animal had more than one blood draw. **Rat AFP Measurements.** Detection and semi-quantitation of rat AFP in sera was by Western blot employing a goat antibody raised against recombinant rat AFP and a standard curve based on AFP from rat amniotic fluid taken at pregnancy day 15. **Measurement of elicited human AFP.** HepG2 cells (10^5 cells/well) were maintained and grown as a monolayer in αMEM (Invitrogen Corporation, Carlsbad, California) supplemented with 5% serum (40% bovine calf serum, 60% FBS), penicillin G (100 units /ml), and streptomycin (100 μg/ml) in 24-well culture plates in triplicates. Cells were changed to serum-free medium when confluent and media were changed

every three days. Hormones, E_3, E_3 + P, E_2 + P or P were dissolved in 95% ethanol, diluted in buffer and brought to 10^{-8} M in the cell culture dish. hCG was dissolved in sterile deionized water and used at a final concentration of 10^{-9}M. Control cells were grown in serum-free medium alone. Hormones were administered daily for 21 days. Media containing secreted AFP were stored at -20°C and were assayed for human AFP on the Beckman-Coulter Access Immunoassay System (Beckman Coulter Inc, Fullerton, CA).

Effect of HepG2 Supernatant on T47D Proliferation. HepG2 cells were grown as above in 25 cm^3 flasks while supplemented with 10^{-9} M hCG daily. Supernatants were collected every third day, centrifuged, concentrated and retained for T47D proliferation experiments. **T47D Cells.** T47D cells (1.2 x 10^5/well) were plated in estrogen-depleted medium and treated with 100 L HepG2 supernatant the day following plating. After incubation for 1 hour, 10 l of 10^{-8} M E_2 (10^{-10}M final concentration) was added. Cells were treated for 7 days with media changes every other day and counted on day 8 using a hemacytometer.

Results

Five groups of carcinogen-exposed rats were treated with different pregnancy-hormone regimens, while a sixth group received no additional treatments. In each case, the reduction of breast cancer incidence in hormone-treated groups is similar to that reported by earlier workers. The decreased incidence is statistically significant for each treatment (compared to control), and multiplicity and tumor volume decreased by each of these treatments.

Rat AFP levels were quantitated using Western blot analysis. Based on 8 replicate studies, treatment with hCG elevated serum AFP concentration 2.4-fold over untreated rat levels, E_2 + P leads to 1.9-fold elevation, E_3 alone produced 2.9-fold elevation, and E_3 + P produced a 3.2-fold rise in AFP levels. Treatment with P alone did not elevate AFP levels persistently (Figure 1).

Fig 1. Measurement of AFP in the sera of Hormone-Treated Animals. Western blot analysis for AFP in rat sera obtained from bleeds at mid-treatment (bleed 2), at conclusion of treatment (bleed 3) and 14 days later (not shown). Controls are rat amniotic fluid (AF), sera from untreated rats (NT) given MNU only, or from male rats. The blot is one of 8 replicate experiments.

To evaluate the effect of hormones on human liver cells, HepG2 cells were incubated with either E_3, E_2 + P, hCG, or P alone, and human AFP released into the cell culture medium was quantified. Figure 2 indicates that treatment with E_3, E_2 + P or hCG led to elevated levels of human AFP above control levels in the media, whereas treatment with P alone did not stimulate AFP production. Control levels (no hormone added to HepG2 cells) ranged from 0.2 to 0.4 ug/ml.

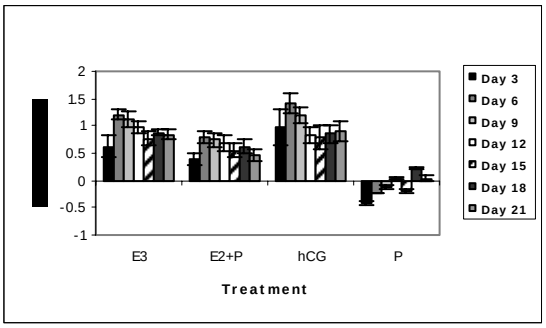

Fig 2. Production of AFP by cultured human HepG2 cells exposed to hormone treatment. AFP levels produced in supernatants of confluent HepG2 cells (10^5/per well) cultured with different hormones were quantified by ELISA and is shown as the difference between the treatment and NT groups.

To assess whether elicited human AFP could inhibit breast cancer cell growth in culture, 10x-concentrated supernatants from hCG- treated HepG2 cells were added to T47D human breast cancer cells growing in culture. As shown in Figure 3, E_2 strongly stimulated growth and the AFP-containing supernatant significantly inhibited it. Similar inhibition of E_2-stimulated growth of T47D cells was produced by a peptide analog of the active site of AFP. In contrast hCG did not directly inhibit T47D cells (data not shown).

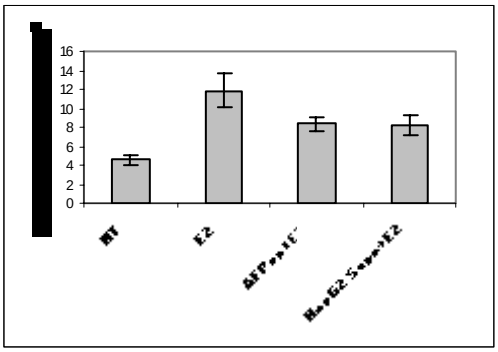

Fig 3. Inhibition of T47D cell proliferation by hCG-treated HepG2 supernatant. T47D cells ($1.2x10^5$) were treated with AFPep (10^{-6}M) or hCG (10^9M)-stimulated HepG2 supernatant (10x) followed by estrogen (10^{-10}M) stimulation for 7 days. Cells were harvested and counted for growth inhibition. *P values (Student's t-test) were 0.006 for inhibition by HepG2 supernatant and 0.01 for AFPep. (n=5).

Discussion

It is clear that treatment of non-pregnant, carcinogen-exposed rats with the hormones of pregnancy leads to a reduction in breast cancer incidence compared to animals exposed only to carcinogen. Our experimental design called for repetition of the prevention studies done earlier, but using MNU as the carcinogen . The incidence of breast cancers and the multiplicity of tumors were very similar to that seen in the earlier studies, and are decreased relative to the carcinogen-only group. Tumor burden, assessed as volume, is likewise reduced

Sera of animals undergoing hormone treatment with significantly reduced breast cancer risk were found to contain rat AFP in concentrations significantly higher than the basal levels in control animals, and this persisted for 14 days following cessation of hormone treatment. A significant control observation was provided by rats treated with P, which fails to provide protection against breast cancer (6). When treated with progesterone (only), they failed to produce sustained high concentrations of AFP. If AFP is the agent of pregnancy responsible for reduction of breast cancer risk, the data suggest that sustained exposure to that protein is required.

It may be questioned whether the cancer prevention experiments in rats constitute a rational extension of the phenomenon occurring in human breast cancer. Since there were no experimental means to elicit AFP from human liver, we undertook a more practical alternative, specifically a challenge of cultured HepG2 human liver cancer cells with these same hormones. With challenges of E_3, E_2 + P, or hCG, the level of human AFP appearing in culture medium was sustained at significantly elevated levels over the basal level secreted by untreated cells. Again, a significant negative control observation was that progesterone alone did not elicit AFP. Using either assay (sera of rats or media from cultured human cells), treatment with the hormones of pregnancy is sufficient to elicit AFP production.

To assess whether elicited AFP is sufficient to attenuate cancer, we used culture media of hCG-stimulated HepG2 (no steroids present) cells to influence the proliferation and response of T47D human breast cancer cells. The culture media of stimulated HepG2 cells is sufficient to inhibit the proliferation of T47D cancer cells. Using an indicator of metabolic response, we also noted that HepG2 media inhibited the estrogen-induced phosphorylation of the estrogen receptor alpha (data not shown).

We postulate that AFP is the pregnancy-associated molecule that leads to reduction of breast cancer incidence later in life. It has been estimated that the doubling time for human breast cancers is about 4 months. Making the rough approximation that growth rate (net mitosis less apoptosis) is constant throughout the duration of an eventual tumor, it can be estimated that to grow from a single cell to a minimally detectable cell mass (approximately10^9 cells) would require 30 doublings, or 10 years. Exposure to the AFP of pregnancy may decrease the mitotic rate relative to apoptosis, leading to tumor extinction, or at least to decreased growth, so that fewer tumors are ever detected. Bennett, et al, (8, 11) have reported on experiments showing human AFP can inhibit the growth of human breast cancer xenografts. It may be possible to capitalize on these observations (*i.e.*, the concept that cancer could be present but undetected for 10 years) in that administration of a safe and effective agent during that window may decrease tumor incidence in humans. An analog of the active site of AFP, termed AFPep, may be such an agent (9). Molecules that mimic E_3, E_2, P or hCG would seem less desirable as preventive agents. If AFP could indeed mimic the chemo preventive effect of pregnancy and were

utilized, then the incidence of breast cancer in the U.S alone would be reduced from 200,000 new cases per year to under 100,000 new cases, possibly below 40,000.

Investigators have given hormones to carcinogen-exposed rats and achieved reduction of breast cancer risk, suggesting that post-carcinogen hormone treatment to reduce breast cancer risk functions by the same mechanism as does pregnancy. This experiment mimics the viewpoint that prolonged exposure to environmental carcinogens occurs prior to pregnancy in humans. However, several investigators have administered the hormone prior to the carcinogen (7), and achieved reduction of breast cancer appearance, which they attribute to hormone-produced changes in the susceptibility of the mammary gland to mutagenic insult. An alternative viewpoint, however, would be that hormones administered prior to the carcinogen produce AFP which, as we have shown, persists sufficiently so as to be present days later, at the time the carcinogen is administered, thus achieving the same result as if the hormones were delivered after the carcinogen.

In summary, it has been clearly shown that breast cancer risk is reduced as a consequence of parity. Definitive studies have shown that the hormones of pregnancy may act independently as surrogates to reduce breast cancer risk in non-pregnant rats. The studies reported herein demonstrate that these treatments also concomitantly elevate AFP levels in non-pregnant adult rat serum. Since this study also shows that AFP inhibits breast cancer growth directly and the hormones of pregnancy do not, it is therefore logical to develop AFP analogs for future chemoprevention of breast cancer.

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Cancer is not a growth of anarchic cells; laws control the survival of the cancer cell and these it must obey. Charles Brenton Huggins, MD, 1979.

Sprague–Dawley rats were obtained from Taconic Farms (Germantown, NY, USA) at 34 days of age and were placed immediately on a controlled diet (Agway Pro-Lab 2000; Agway Corporation, Syracuse, NY, USA), allowed free access to food and water, and maintained on a 12-h light–dark cycle at a constant temperature (22°C) for the duration of the study. Severe combined immune-deficient (SCID) mice and Swiss Webster mice were obtained from Taconic Farms and were maintained in individually ventilated cages. Cages, bedding, food, and water for mice were autoclaved. Mice were handled using sterile technique in a laminar flow biosafety cabinet. All animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at Albany Medical College who are guided by the United States Public Health Service regulations on the humane care and use of laboratory animals, and these guidelines meet the standards required by the UKCCCR (Workman *et al*, 1998).

Peptide synthesis

The AFP-derived peptide, *cyclo*(EKTOVNOGN), where O is hydroxyproline, was generated using FMOC solid-phase peptide synthesis employing the head-to-tail cyclisation method (Kates *et al*, 1993). After synthesis, the resin was washed with propanol and partially dried, and peptides were cleaved from the solid support and deprotected simultaneously with 10 ml of trifluoroacetic acid/thioanisole/anisole/ethanedithiol (90 : 5 : 2 : 3) per 0.5 g of resin for 5 h. Peptide was recovered from the liquid phase after repeated extraction, first with ether and then with ethyl acetate/ether (1.5 : 1). The peptide was dissolved in water, purified by reverse-phase HPLC, and then lyophilised. Biologically active AFPep can also be purchased from PolyPeptide Laboratories (Torrance, CA, USA) after synthesis by the tBOC method, or from Advanced ChemTech (Louisville, KY, USA) after synthesis by the FMOC method.

Cell culture assay

The MCF-7 cells were maintained in monolayer culture in Dulbecco's modification of Eagle's medium (DMEM) supplemented with 5% fetal bovine serum, glutamine (2 mM), nonessential amino acids (1%), and bovine insulin (1 $\mu g\,ml^{-1}$). The T47D human breast cancer cells were maintained in monolayer culture in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 IU ml^{-1} penicillin, 100 $\mu g\,ml^{-1}$ streptomycin, 0.25 $\mu g\,ml^{-1}$ amphotericin B, and 8 $\mu g\,ml^{-1}$ bovine insulin in T-75 flasks with 2–3 medium changes per week. Cells were maintained at 37°C in an atmosphere of 5% CO_2, 95% air. To evaluate the oestrogen-stimulated growth of these cells, they were released from monolayer using 0.25% trypsin/0.53 mM EDTA and suspended in oestrogen-free medium comprised of DMEM (high glucose, phenol red-free), supplemented with 10% charcoal-stripped bovine calf serum, 2 mM glutamine, 100 IU ml^{-1} penicillin, and 100 $\mu g\,ml^{-1}$ streptomycin. One millilitre containing 1.2×10^5 cells was seeded into each well of a 24-well plate coated with collagen IV (BD Biosciences, Bedford, MA, USA). Beginning from 1 day after seeding, cells were treated daily for 7 days with AFPep (10^{-6} M), Tam (10^{-8} M), or the combination of AFPep plus Tam in the presence or absence of trans E_2(10^{-9} M). In these groups exposed to E_2, E_2 at 10^{-9} M was added 1 h after each addition of AFPep and Tam. Control wells received the vehicle. Medium was changed every other day before treatment. One day after the last treatment, cells were trypsinised and resuspended in oestrogen-free medium, and counted using a haemacytometer following dilution with trypan blue. Viable cell number is reported.

Binding to oestrogen receptor

Rabbit uteri (Pel-Freez Biological, Rogers, AR, USA) were used as a source of ER. Uteri were pulverised in a stainless-steel impact mortar under liquid nitrogen and homogenised (20% w/v) in buffer (10 mM Tris, pH 7.4, 1.5 mM EDTA, 10% glycerol, 10 mM monothioglycerol, and 10 mM sodium molybdate) on ice. Centrifugation (50 000 **g**) for 1 h yielded cytosol, which was adjusted with buffer to 2.5 mg protein ml^{-1}. All incubations were carried out in triplicate, each containing 100 μl of cytosol, 20 μl of 10 nM 6,7-[^{3}H]oestradiol (6,7-[^{3}H]E_2), and 80 μl of antagonist. After incubation overnight at 4°C, tubes received 300 μl of dextran-coated charcoal suspension; tubes were agitated for 15 min, and then centrifuged (1000 **g**) for 15 min. Supernatants were decanted into counting vials, scintillation fluid was added, and protein-bound tritium was determined (Mesfin *et al*, 2001).

Xenograft assay

Ten million MCF-7 human breast cancer cells were harvested from culture, centrifuged into a pellet, solidified into a fibrin clot, and implanted under the kidney capsule of SCID mice as described previously (Bennett *et al*, 1985, 2002, 2006; Mesfin *et al*, 2001; Parikh *et al*, 2005). Oestrogen supplementation was accomplished by s.c. implantation of a 5 mm silastic tubing capsule containing solid E_2 inserted on the day of tumour implantation. One milligram of Tam was dissolved in 1 ml 95% ethanol and then diluted to 5 and 0.25 $\mu g\,ml^{-1}$ in saline; 0.2 ml of these concentrations was administered to mice. α-Fetoprotein-derived peptide was dissolved directly in saline to its appropriate concentrations. Tam was administered p.o. by gavage and AFPep was administered i.p. once a day beginning from 1 day after tumour implantation. The tumour-bearing kidney was exposed during survival laparotomy at 14 and 28 days after implantation and tumour size was measured using a dissecting microscope equipped with an ocular micrometer, noting the long (D) and short (d) diameters of the tumour. Five replicate mice were included in each treatment group. Mean tumour volume ($0.52d^2D$) was calculated for each group, and used for display of growth curves. Significance of differences between groups was tested using the one-sided Wilcoxon rank sum test.

Prevention assay

The prevention study utilised methodologies previously described (Grubbs *et al*, 1985; Parikh *et al*, 2005) to test the ability of AFPep, Tam, or the combination of AFPep and Tam to prevent MNU-induced breast cancers in rats. There were 30 rats in each experimental group to assure a 95% probability of detecting a difference between groups (ratios) of 40%, which was the difference seen for pregnancy (Grubbs *et al*, 1983, 1985, 1986; Swanson *et al*, 1997). Power analysis was performed by SOLO software, BMDR Statistical Software Inc. (Los Angeles, CA, USA). Female rats were housed three per cage in a room maintained at 22 ± 1°C, and artificially lighted for 12 h per day. At 50 days of age, rats received a single injection of MNU (50 mg kg^{-1} body weight) or vehicle in the jugular vein. *N*-methyl-*N*-nitrosourea was administered to animals in the various treatment groups according to a predetermined randomisation chart, so as to ensure uniform distribution of the carcinogen across the groups. Beginning 10 days after MNU exposure, treatment with AFPep by s.c. injection, or Tam by oral gavage, occurred once daily. These routes were selected based on prior experience with these drugs in animals (Bennett *et al*, 2002) and potential routes for these drugs in women. To cause minimal stress to the animals, the route control manipulation (i.e., vehicle injection in Tam animals and vehicle gavage in AFPep animals) was not included. The peptide was diluted in saline and 0.2 ml was administered s.c. for 20 days, while Tam was dissolved in corn oil and was administered by oral gavage

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(0.2 ml) for the same time period. A control group of animals received daily 0.2 ml s.c. injections of saline for the same time period as AFPep administration and experienced the maximal number of tumours. Starting 30 days after MNU treatment, all rats were palpated weekly for detection of mammary tumours, noting number, location, and size. Tumour burden was determined noninvasively using calipers to measure the long (D) and short (d) diameter of each tumour. Assuming that tumours were ellipsoid shaped, tumour volume was estimated as ($0.52d^2D$). Cage activity of all animals was monitored daily for gross signs of toxicity. At necropsy, body weight and organ weights were assessed as indicators of toxicity.

Toxicity

To evaluate the effect of AFPep and Tam on uterine hyperplasia, the immature mouse uterine growth assay was utilised (Mesfin *et al*, 2001; Bennett *et al*, 2002; Parikh *et al*, 2005). Briefly, various doses of AFPep and/or Tam were injected i.p. into 2-week old female Swiss pups. Control mice received the vehicle. Twenty-two hours later, uteri were harvested, trimmed free of connective tissue, and weighed. Uterine weights were normalised to donor mouse body weight. There were five replicate mice per group.

In the therapy model (human tumour xenografts growing in mice) and in the prevention model (MNU-induced mammary cancers in rats), the toxicity of AFPep, Tam, and AFP plus Tam was evaluated by monitoring animal body weights, fur textures and cage activity, and weights of specified organs at necropsy.

RESULTS

As shown in Figure 1A, either Tam or AFPep when used alone inhibited the E_2-stimulated growth of T47D human breast cancer cells in culture. The combination of Tam plus AFPep demonstrated cooperative growth inhibition as exemplified in Figure 1B in which IC_{40} values of Tam plus AFPep produced an 80% inhibition of E_2-stimulated breast cancer growth. This is especially important when one considers that the IC_{40} of Tam in these experiments was approximately 100-fold lower than the IC_{80} of Tam, suggesting that combination of Tam with AFPep would permit using a lower dose of Tam without overall loss of antitumour activity. Additional results described in subsequent figures support this important concept. In data not shown, it was apparent that, at concentrations ranging from 10^{-8} to 10^{-5} M, neither Tam nor AFPep inhibited the basal (no E_2) growth of T47D cells, suggesting that their action was directed mainly to the E_2 stimulation of these cells and was not a nonspecific toxic effect. Also, a control peptide of scrambled sequence did not inhibit the E_2-stimulated or basal growth of these cells (Bennett *et al*, 2006)

Although T47D cells were quite responsive to E_2 in cell culture, these cells were less reliable when grown as a xenograft in immune-deficient mice, having a take rate (i.e., successful growth) of $<60\%$ when implanted in 20 of these mice. In contrast, we have found the MCF-7 human breast cancer cell line to have a tumour take rate of 100% in immune-deficient mice and to be completely dependent on E_2 for growth in these mice. Hence, MCF-7 xenografts were used as a model of E_2-dependent human breast cancer being treated with AFPep and Tam *in vivo*. Again, several doses of these drugs were evaluated in preliminary studies in this model to find optimal and suboptimal doses, which were then studied alone and in combination in the studies described herein. As shown in Figure 2A, AFPep at a dose of 10 μg mouse^{-1} day^{-1} or Tam at a dose of 1 μg mouse^{-1} day^{-1}, completely prevented the growth of MCF-7 tumour xenografts over a 30-day period. When doses of AFPep or Tam were lowered to 0.1 and 0.05 μg per mouse per day, respectively, tumour growth occurred, although it was less than tumour growth in nontreated mice (Figure 2B). Interestingly,

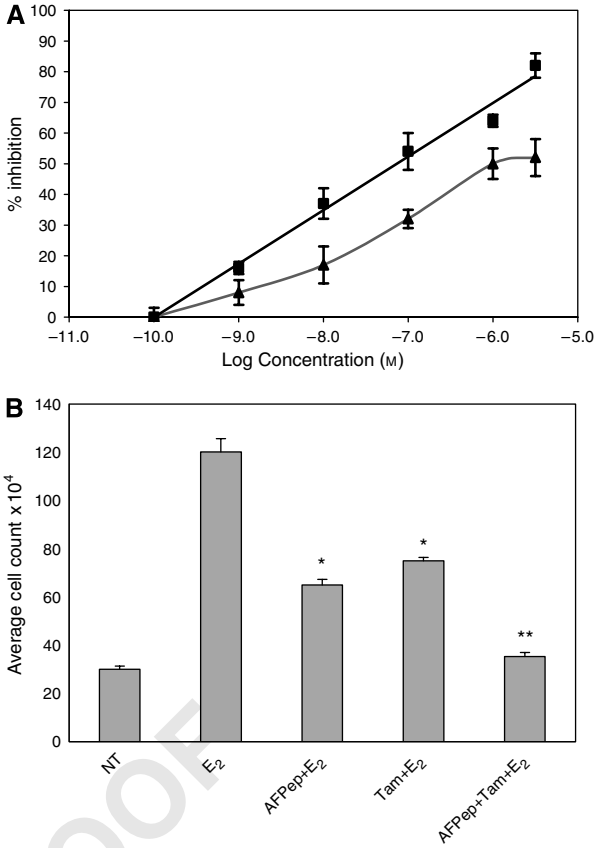

Figure 1 Inhibition of E_2-stimulated growth of T47D human breast cancer cells by AFPep and Tam. T47D (1×10^5) cells were seeded into wells of collagen-coated plates in oestrogen-free medium. Medium was changed daily. Twenty-four hours after seeding, AFPep and/or Tam were added. One hour later E_2 (10^{-9} M) was added. Cells were treated daily for 7 days after which cells were harvested and counted in a hemacytometer. Mean viable cell number of six replicate wells $\pm$s.e. was determined. (**A**) Concentration–response curve of each agent alone. Inhibition (%) of E_2-stimulated growth is reported. (■) Tam; (▲) AFPep. (**B**) Effect of AFPep and/or Tam on T47D cell proliferation. Mean viable cell number is reported. E_2, 10^{-9} M; AFPep, 10^{-6} M; Tam, 10^{-8} M (NT, no treatment). *$P<0.05$ *vs* E_2 alone, Dunnett's test. **$P<0.05$ *vs* Tam alone, Scheffe's test.

when AFPep and Tam were combined at these suboptimal doses, MCF-7 tumour growth was completely prevented (Figure 2B), again indicating that the maximal effect of Tam can be achieved at a much lower dose of Tam, if it is combined with AFPep. The validation of previous studies (Jacobson *et al*, 1990; Bennett *et al*, 1998, 2002; Mesfin *et al*, 2001; Parikh *et al*, 2005), which have shown that MCF-7 tumours do not grow without E_2 supplementation is not shown in Figure 2.

As a model for preventing the development of breast cancer, we utilised the well-characterised system of carcinogen (MNU)-induced mammary cancer in Sprague–Dawley rats (Grubbs *et al*, 1983, 1985, 1986; Swanson *et al*, 1997). When carcinogen-exposed rats were treated with Tam at doses of 6.25 μg animal^{-1} day^{-1} or higher, or AFPep at 270 μg animal^{-1} day^{-1}, there was significant inhibition of tumour formation, as shown in Table 1. These results are similar to those of Moon *et al* (1992, 1994) using Tam and to Parikh *et al* (2005) using AFPep. At a suboptimal dose of Tam or of AFPep, inhibition of tumour formation was not significantly diminished compared to controls (Table 1, Figure 3). When these two drugs were used in combination at suboptimal doses, their combined chemopreventive contribution resulted in a decrease in

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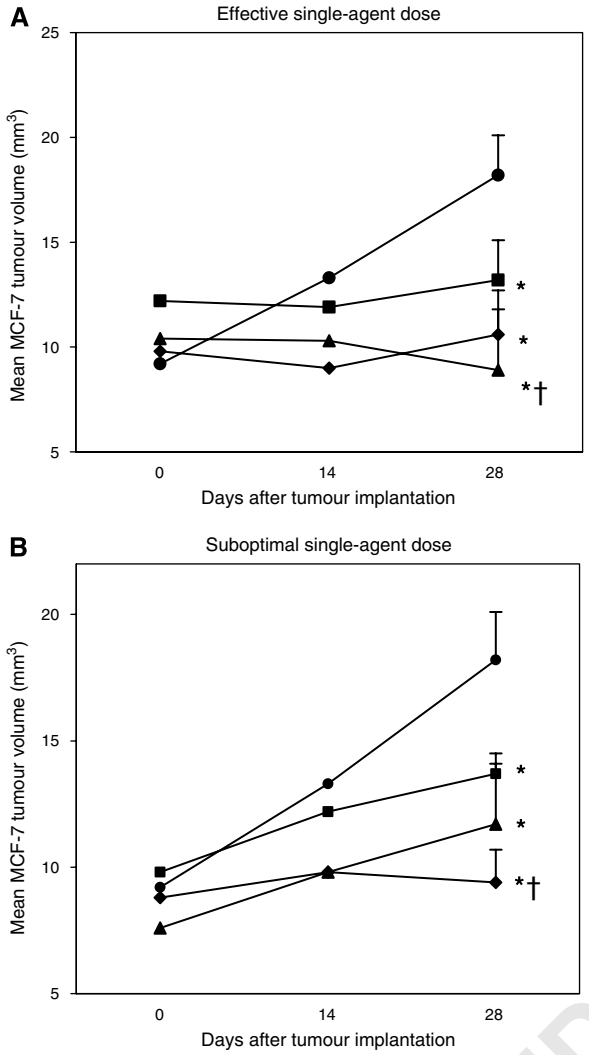

Figure 2 Inhibition of E_2-stimulated growth of MCF-7 human breast cancer xenografts by AFPep and Tam. Pieces of MCF-7 tumour were implanted under the kidney capsule of SCID mice. Oestradiol was provided via an E_2-containing silastic tubing subcutaneous implant. Vehicle (●), AFPep (▲), Tam (■), or AFPep + Tam (◆) were injected once daily at the doses indicated below beginning from 1 day after tumour implantation. (**A**) Effective single-agent dose (10 μg AFPep and 1 μg Tam). (**B**) Suboptimal single-agent dose (0.1 μg AFPep and 0.05 μg Tam). Mean tumour volume of five replicate mice ± s.e. is reported. *$P <$ 0.05 *vs* vehicle; †$P < 0.05$ *vs* Tam alone, Wilcoxon rank-sum test.

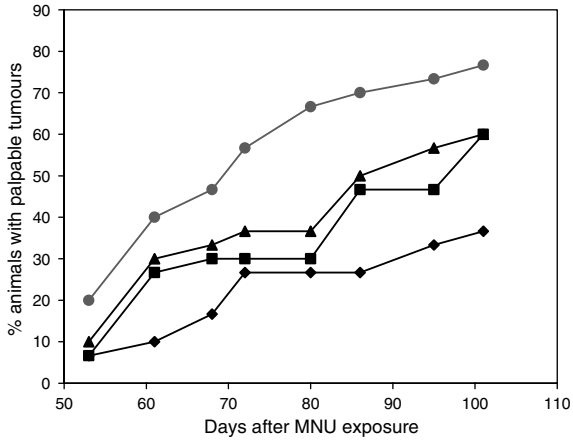

Figure 3 Combination of suboptimal doses of AFPep and Tam prevents breast cancer. Sprague–Dawley female rats ($n = 30$ rats group^{-1}) received MNU (50 mg kg^{-1}) at the age of 50 days. Beginning after 10 days, rats were treated once daily with vehicle (●), or a suboptimal dose of Tam (■, 50 ng animal^{-1}, p.o.), or with a suboptimal dose of AFPep (▲, 100 μg animal^{-1}, s.c.) once daily for 20 days, or with both (◆). Animals were palpated weekly. The incidence of tumours is shown as a function of time after carcinogen treatment.

tumour incidence (Figure 3, Table 1), which was significantly below that seen in control ($P = 0.04$, Fisher's exact). Doses of AFPep (100 μg animal^{-1} day^{-1};) or of Tam (0.05 μg animal^{-1} day^{-1}) given to the rats were held constant during the 20-day treatment interval. During this interval, all animals in all groups gained weight from 165 to 190 g, indicating that treatments did not affect body weight. Table 1 shows that latency increased significantly, multiplicity decreased, and tumour burden (volume) decreased significantly following combination treatment with the lower doses of AFPep in combination with Tam.

In all previous work with high doses of AFPep, no evidence of toxicity or side effects has been detected (Mesfin *et al*, 2001; Bennett *et al*, 2002, 2006; Parikh *et al*, 2005). In this study in which AFPep or combination of AFPep and Tam were administered to rats at therapeutic doses for 2–3 weeks, there was no effect on body weight, fur texture, or cage activity during the lifespan of the animals, or on organ weights obtained at necropsy (Table 1). At these low doses, Tam had no effect on uterine growth, whether or not AFPep was present.

A side effect of Tam is the induction of uterine hyperplasia in approximately 30% of the patients taking this drug, and this progresses to uterine cancer in roughly 0.2% of users (Fisher *et al*, 1994; Assikis and Jordan, 1995). As shown in Figure 4, Tam stimulates the growth of immature mouse uterus. α-Fetoprotein-derived peptide does not stimulate uterine growth. Moreover,

Table 1 Effect of AFPep and Tam on prevention of breast cancer in MNU-exposed rats

Treatment	Dose (μg/rat/day)	Incidence,[a] % (*P*-value[b])	Multiplicity[c] (tumours/rat)	Latency[d] (days)	Volume[e] (cm^3)	Body weight, g	Uterine weight (g)	Heart weight (g)
None (control)	—	78	2.1	80±31	69.0	283±30	0.54±0.15	1.10±0.14
AFPep	270	40 (0.02)	0.5	88±16	34.5*	280±29	0.62±0.17	1.09±0.13
AFPep	100	63 (0.16)	1.1	97±33	39.1*	285±24	0.52±0.15	1.12±0.14
Tam	6.25	26 (0.001)	0.5	85±15	70.2	273±28	NA	NA
Tam	0.05	73 (0.21)	1.3	95±36	62.4	282±31	0.49±0.17	1.12±0.13
AFPep±Tam	100±0.05	52 (0.04)	0.9	110±33*	32.6*	282±29	0.51±0.12	1.09±0.15

AFPep = α-fetoprotein-derived peptide; MNU = *N*-methyl-*N*-nitrosourea; NA = not applicable; Tam = tamoxifen. *$P < 0.05$ *vs* control. [a]Percent of rats with one or more tumours when killed (approximately 120 days after MNU exposure). [b]*P*-value (calculated according to Fisher's exact test) compared to control group. [c]Multiplicity is defined as total number of tumours/number of rats. [d]Mean number of tumour-free days ± s.d. [e]Sum of the volumes of tumours in each group.

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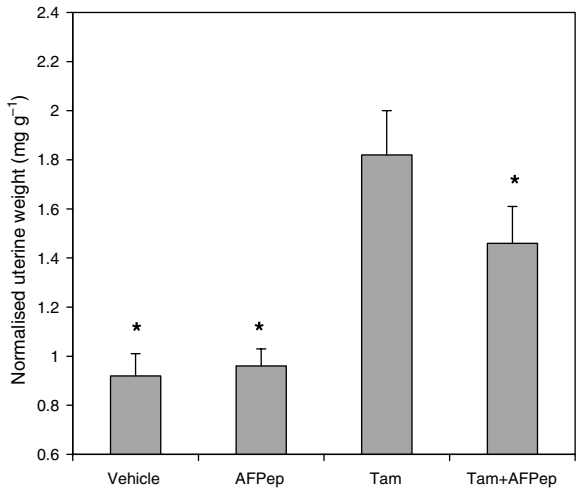

Figure 4 Effect of AFPep and Tam on growth of immature mouse uterus. Mice were injected i.p. with Tam (1 μg), AFPep (10 μg), or AFPep plus Tam at the doses already indicated. In the case of the combination, AFPep was given 1 h before Tam. Twenty-two hours after treatment, uteri were harvested, weighed, and normalised to mouse body weight. Mean uterine weight of five replicate mice per group $\pm$s.e. is reported. *$P<0.05$ *vs* Tam alone, Dunnett's test.

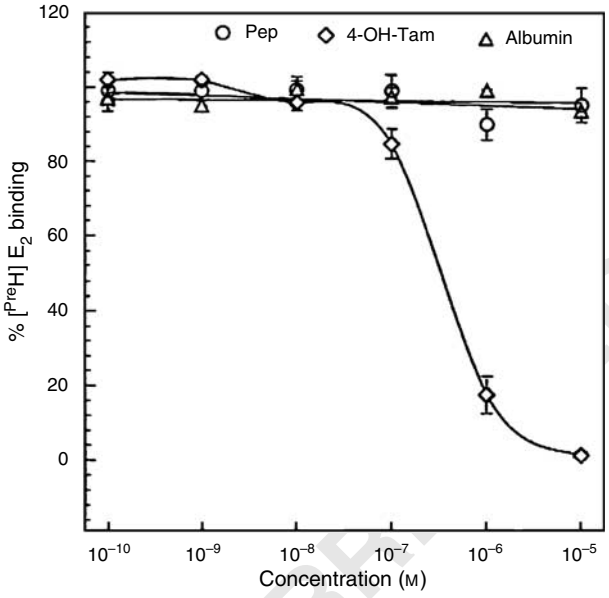

Figure 5 Effect of AFPep and Tam on binding of E_2 to the ER. Rabbit uterine cytosol was used as a source of ER. Test agents (80 μl) at the final concentrations indicated were incubated in triplicate with 100 μl of cytosol, and 20 μl of 10 nM 6,7-[^{3}H]E_2 (50 Ci mmol^{-1}). The concentration of the [^{3}H]E_2 complex with receptor in the presence of the test agent is expressed as a percentage of the amount of complex formed in the absence of test agent.

AFPep significantly inhibits the Tam-induced growth of the immature mouse uterus. Therefore, in women, adding AFPep to Tam would be expected to diminish the uterine growth side effect of Tam.

Mechanistically, AFPep is quite different from Tam. As shown in Figure 5, AFPep did not inhibit the binding of [^{3}H]E_2 to ER, while Tam demonstrated the concentration-dependent inhibition of E_2 binding expected from an ER antagonist. Also, in other studies, we have shown that AFPep did not stimulate phosphorylation of ER at serine 118 (Bennett *et al*, 2006), which is the phosphorylation site stimulated by E_2 (Kato *et al*, 1995). In contrast, it has been shown that Tam stimulates phosphorylation of ER at serine 118, following its binding to this receptor (Chen *et al*, 2002). Thus, Tam and AFPep impact the ER in quite different ways, and yet work cooperatively together to inhibit oestrogen-dependent breast cancer growth.

DISCUSSION

Tamoxifen has been a very effective drug for the treatment of ER+ breast cancer (Lippman and Bolan, 1975; Early Breast Cancer Trialists' Collaborative Group, 1992; Fisher *et al*, 1998; Jordan, 1999a). However, its effectiveness wanes with time, and after 2–5 years of treatment, most ER+ tumours become refractory to Tam (Early Breast Cancer Trialists' Collaborative Group, 1992; Jordan, 1999a). Also, there are some ER+ breast cancers that are unresponsive to Tam at first presentation (Jensen and DeSombre, 1996), and there are others that, after chronic treatment with Tam, actually become growth-stimulated by Tam (Canney *et al*, 1987; Gottardis and Jordan, 1988; Howell *et al*, 1992). There are no good tests to differentiate between these phenotypes, and it is possible that seeds of each phenotype are present when breast cancer is first diagnosed. One logical approach to this problem is to treat with combinations of anti-endocrine agents using the prescribed combination principles of each agent being active alone, having different mechanisms of action and having non-cross-reacting host toxicities. With this strategy, those phenotypes not held in check by Tam would theoretically be arrested by the agent(s) combined with Tam. Zaccheo *et al* (1991, 1993) have validated this principle by showing that Tam plus examestane, an aromatase inhibitor, was more effective than Tam alone in stopping breast cancer growth, and now combinations of Tam plus aromatase inhibitors are showing promise clinically (Abrial *et al*, 2006).

The data in the study reported herein support three independent concepts: AFPep has antitumour activity on its own, it adds to the antitumour activity of Tam through a mechanism distinct from Tam, and it can reduce the toxicity of Tam by decreasing the uterine hyperplasia of Tam and by allowing dose reduction of Tam without loss of antitumour activity.

We have been studying AFPep as an inhibitor of oestrogen-dependent growth (Mesfin *et al*, 2001; Bennett *et al*, 2002; Parikh *et al*, 2005). α-Fetoprotein-derived peptide is not an ER antagonist, nor an ER partial agonist, making its mechanism different from Tam (Parikh *et al*, 2005; Bennett *et al*, 2006). Its function, at least in part, is to inhibit the phosphorylation of ER that follows ligand binding to ER. Phosphorylation has been shown to be necessary for full functionality of ER (Kato *et al*, 1995). Furthermore, AFPep does not share the toxicities of Tam. In fact, in this study and in studies reported elsewhere (Bennett *et al*, 2002), AFPep interferes with the uterine hyperplasia induced by Tam (i.e., reduces the toxicity of Tam). The fact that AFPep inhibits Tam stimulation of growth in the uterus suggests that AFPep may inhibit not only that toxicity of Tam in humans but also those breast cancer phenotypes that are actually stimulated by Tam, as well as those that are indifferent to Tam. α-Fetoprotein-derived peptide clearly fits the combination principles of being active when used alone, having a different mechanism of action from Tam, and having non-cross-reacting host toxicity with Tam. Hence, it was eminently logical to postulate that AFPep would be beneficial in combination with Tam, and the results of this study provide the data to support this contention. Not only did AFPep plus Tam inhibit the growth of an extant oestrogen-dependent human breast cancer better than Tam alone, but also the combination was more effective than Tam alone in preventing carcinogen-induced mammary cancers in rats. It should not go unnoticed that in the effective combination, the dose

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of Tam was substantially lower than that employed for Tam alone, suggesting that such dose reduction may, in itself, alleviate Tam's toxicities (Jordan, 1999b). In addition, the data in Figure 4 suggest that AFPep will further alleviate some of those toxicities as it inhibits Tam-induced uterine hyperplasia. No toxicities from AFPep have become evident. Since it is derived from a natural human fetal protein (α-fetoprotein, AFP), and since its active dose is below the fetal physiological level of AFP, it is possible that the side effects associated with AFPep will, at most, be minimal. Thus, it is highly unlikely that it will add to any of the toxicities of Tam, and, as mentioned above, should alleviate some of those toxicities while contributing to the antitumour activity of Tam. While the study reported herein was ongoing, it was found that AFPep was active by the oral route (Bennett *et al*, 2006). Hence, the discomfort of chronic injections will not be an issue with use of AFPep as a therapeutic or preventive agent.

Using a variety of models and species (human breast cancer cells growing in culture, human breast cancer cells growing in a an immune-deficient mouse model of therapy, or chemically induced mammary carcinoma in a rat prevention model), the results of this study have shown that AFPep works well in combination with Tam for the treatment and prevention of experimental breast cancer. Its mechanism is quite different from that of Tam, and its toxicity is minimal. As such, AFPep warrants further development as a new agent that could be used in combination with Tam, or perhaps even used as a stand-alone agent, for the treatment or prevention of breast cancer in humans.

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